EXHIBIT G

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Hedgehog Controls Limb Development by Regulating the Activities of Distinct Transcriptional Activator and Repressor Forms of Cubitus interruptus

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Summary

Hedgehog (Hh) proteins play diverse organizing roles in development by regulating gene expression in responding cells. The Gli homolog Cubitus interruptus (Ci) is involved in controlling the transcription of Hh target genes. A repressor form of Ci arises in the absence of Hh signaling by proteolytic cleavage of intact Ci. We show that this cleavage is essential for limb patterning and is regulated by Hh in vivo. We provide evidence for the existence of a distinct activator form of Ci, which does not arise by mere prevention of Ci proteolysis, but rather depends on a separate regulatory step subject to Hh control. These different activities of Ci regulate overlapping but distinct subsets of Hh target genes. Thus, limb development is organized by the integration of different transcriptional outputs of Hh signaling.

Introduction

Some of the most powerful morphogenetic signaling molecules implicated in a broad variety of developmental patterning processes are members of the Hedgehog (Hh) family of secreted proteins (reviewed by Hammerschmidt et al., 1997). Their function and mode of action is best understood for *Drosophila* limb development where Hh controls the expression of the long-range morphogens Wingless (Wg) and Decapentaplegic (Dpp; reviewed by Lawrence and Struhl, 1996; Neumann and Cohen, 1997). Each leg and wing primordium is subdivided into two cell populations, the anterior (A) and posterior (P) compartments. Cells in one compartment, P, are programmed by the selector gene *engrailed* (*en*) to secrete Hh. *en* is not active in A compartment cells, and as a consequence, these cells are primed to receive the Hh signal (reviewed by Lawrence and Struhl, 1996).

The ability of Hh to move into the A compartment and induce a thin stripe of Dpp expression next to the A/P compartment boundary depends on a novel transduction mechanism that involves the cell surface proteins Patched (Ptc) and Smoothened (Smo; reviewed by Alcedo and Noll, 1997; Ingham, 1998). Ptc is expressed in all A compartment cells and, in the absence of Hh, inbibits the activity of Smo that is essential for Hh signal transduction. Binding of Hh to Ptc releases latent Smo activity, activating the transduction pathway. Activity of the pathway induces *ptc* as well as *dpp* expression, and the high levels of Ptc that accumulate on the surface of A compartment cells appear to restrict the further movement of Hh into the A compartment (Chen and Struhl, 1996).

The downstream-most component known in the Hh signal transduction pathway is the transcription factor Cubitus interruptus (Ci), the *Drosophila* Gli homolog (Orenic et al., 1990). Ci is expressed exclusively in A compartment cells and accumulates in the cytoplasm of anterior cells along the A/P boundary, where genes such as *ptc*, *dpp*, and *wg* are transcribed at high levels in response to Hh signaling (reviewed by Alcedo and Noll, 1997; Ingham, 1998). Away from the boundary, cells are not exposed to Hh, and Ci is proteolytically cleaved to yield a lower molecular weight product, the N-terminal fragment Ci-75. This truncated form of the protein localizes to the nucleus, where it appears to function as a transcriptional repressor (Aza-Blanc et al., 1997; Hepker et al., 1997).

It has been suggested that Hh target genes, such as *ptc* and *dpp*, are directly repressed in the absence of Hh signaling by the Ci-75 protein (Aza-Blanc et al., 1997). A repressive role for Ci in transcription is also supported by clonal analysis, since removal of *ci* activity led to ectopic expression of *hh* and *dpp* (Dominguez et al., 1996). In contrast, the accumulation of full-length Ci in response to Hh signaling is associated with the transcription of Hh target genes. Stabilization of the intact protein might activate these genes because full-length Ci could function as a transcriptional activator. Some Hh target genes, such as *ptc* and *wg*, contain Ci-binding sites and have been shown to be transcriptionally activated in the presence of Ci in yeast or tissue culture cells (Alexandre et al., 1996; Von Ohlen and Hooper, 1997). Moreover, overexpression of Ci in vivo can result in ectopic expression of *wg*, *ptc*, and *dpp* in a Hh-independent manner (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997). However, it is equally plausible that the expression of Hh target genes is upregulated by the reduction of Ci-75 repressor levels in response to Hh signaling. Previous studies have not resolved this issue.

Here we address which mechanism—transcriptional activation or derepression—governs target gene expression by Ci in response to Hh. To do this, we have established a genetic system in which three states of Ci are analyzed: (1) no activity, (2) only repressor activity, and (3) only activator activity. We find that intact Ci acts as a transcriptional activator. In contrast, a truncated form of Ci similar to Ci-75, at physiological levels, acts as a transcriptional repressor. Using a form of Ci that is resistant to proteolytic cleavage, we provide evidence that cleavage is required to generate Ci repressor activity and that Hh signaling is necessary for the full-length form of Ci to function as activator. Surprisingly, the Ci activator and repressor forms regulate the expression of different subsets of Hh target genes. In the wing, *ptc* expression, as well as a late phase of Hh-dependent *en* expression, is controlled solely by the Ci activator function, whereas *hh* is regulated only by the repressor

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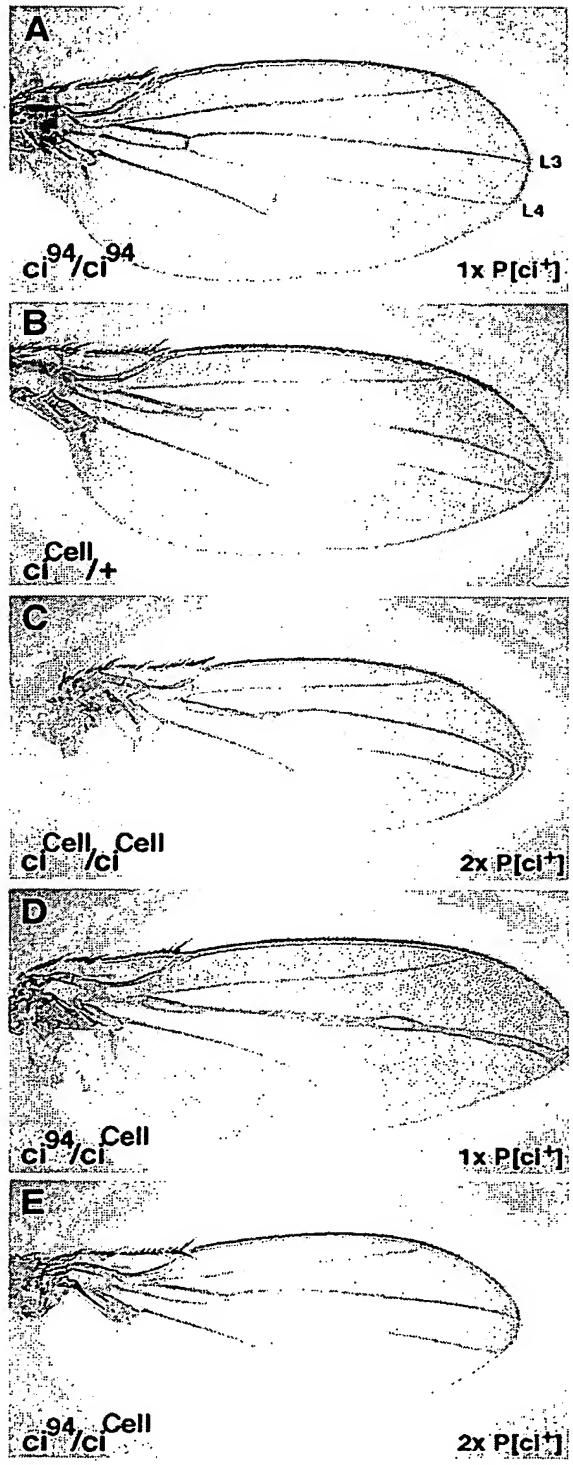

Figure 1. Rescue of ci^{94} and ci^{Cell} Mutants by a Transgene Containing All Essential Elements of *ci*

Wing phenotypes of animals expressing different levels of mutant Ci^{Cell} and wild-type Ci protein are shown. In all panels, anterior is up and distal to the right.

(A) Complete rescue of homozygous ci^{94} null mutant animals by a single copy of *P[ci+]*. (B) Wing of a ci^{Cell}/+ animal with partial fusion of longitudinal veins 3 (L3) and 4 (L4) in the distal and proximal regions of the wing and concomitant loss of some L3/4 intervein material. (C) Homozygous ci^{Cell} animal rescued by two copies of *P[ci+]*. (D and E) Wings of ci^{Cell}/ci^{94} animals rescued by one (D) or two (E) copies of *P[ci+]*. Comparison of (C) and (D) indicates that, although the ratios of ci^{Cell} to *P[ci+]* copies are the same in the genotypes shown, the fusion between L3 and L4 is less severe if two copies of *P[ci+]* are present. This suggests that in a situation in which the levels of Ci activator and repressor forms increase concomitantly (identical ratio, but increased absolute difference), Ci activator activity dominates over Ci repressor activity.

activity. By contrast, both the domain and levels of transcription of *dpp*, which ultimately determine the size and pattern of the adult appendage, are controlled by the balance between the Hh-dependent activator and repressor activities of Ci.

Results

Although *ci* was identified more than 60 years ago, analysis of its role has been severely hindered by two obstacles. First, the *ci* gene is located on the fourth chromosome, imposing major technical difficulties in studying its role during development. Second, no bona fide null allele has been available. ci^{D}, one of the founding alleles that has been used extensively for the genetic characterization of the locus, alters the activity of *ci* and abolishes that of an adjacent gene, *pangolin/Dlef1*, which plays an essential role in Wg signal transduction (see Schweizer and Basler, 1998). Here we have overcome these difficulties by identifying a true null allele and generating genomic transgenes that carry all or only some of the activities of the ci^{+} locus inserted at other chromosomal locations, enabling the generation of mutant cell clones by Flp-mediated mitotic recombination (Xu and Rubin, 1993).

Identification of a Null Allele of *ci*

A candidate null allele of *ci* is ci^{94}. This mutation was generated by the imprecise excision of a P element located in the promoter region of the *ci* gene (Slusarski et al., 1995). Homozygous ci^{94} mutant embryos die and display a segment polarity defect with a deficit of naked cuticle (Slusarski et al., 1995). We determined the molecular lesion of ci^{94} by sequence analysis and found it to contain a deletion of 5 kb, from nucleotides 10,997 to 15,927 (numbering according to Ahmed and Podemski, 1997). This deletion removes the promoter and the first exon of *ci* and consequently the sites for transcriptional and translational initiation. Thus, ci^{94} represents a bona fide null allele of *ci*.

ci^{Cell} Encodes a Truncated Form of Ci that Acts as a Constitutive Transcriptional Repressor

A candidate allele of *ci* that may code for a repressor form of Ci is ci^{Cell-2} (here referred to as ci^{Cell}). This allele gives rise to a protein that is smaller than wild-type Ci and accumulates to high levels throughout the anterior

compartment of wing imaginal discs (Slusarski et al., 1995). We found that ci^{Cell} differs from wild-type *ci* by an 8 bp deletion that is expected to result in a truncation of the Ci protein product at amino acid 975. Expression of a transgene encoding Ci^{Cell} represses transcription of Ci target genes in the wing imaginal disc (see below). These findings and the dominant phenotype associated with ci^{Cell} (Figure 1B) suggest that the mutant protein acts as a constitutive inhibitor of Hh target gene expression.

Rescue of ci^{94} and ci^{Cell} by a ci^{+} Transgene

Homozygous ci^{94} animals are rescued to adulthood by one copy of a ci^{+} transgene that contains 16 kb of the *ci* locus (see Experimental Procedures). Rescued flies are healthy, fertile, and exhibit no obvious phenotypes (Figure 1A). In contrast, ci^{Cell} homozygous animals could not be rescued by one copy of the genomic construct, but survived to late pupal stages with rare adult escapers when two copies were present (Figure 1C). This result is consistent with the interpretation that Ci^{Cell} functions in a dominant-negative manner. Indeed, a single ci^{Cell} allele (in ci^{Cell}/ci^{94} animals) is rescued with one copy of the ci^{+} transgene. Wings of such animals exhibit an extreme form of the dominant ci^{Cell} phenotype, that is, a fusion of the longitudinal veins L3 and L4 (Figure 1D). This phenotype is absent in ci^{Cell}/ci^{94} animals rescued by two copies of the ci^{+} transgene (Figure 1E).

Ci Functions as a Repressor of *hh* Transcription

Clones of cells homozygous mutant for ci^{94} (and thus completely lacking any Ci product) were generated by Flp-mediated mitotic recombination using the ci^{+} transgene (see Experimental Procedures). ci^{-} clones located in the A compartment ectopically express a *hh-lacZ* reporter gene, albeit at levels lower than those of endogenous *hh-lacZ* expression in P compartment cells (Figure 2A). By contrast, clones of A compartment cells mutant for ci^{Cell} (ci^{Cell}/ci^{94} clones) do not express the *hh-lacZ* gene (Figure 2B). These results show that Ci is normally required in A compartment cells to repress *hh* transcription and that physiological levels of Ci^{Cell} (from a single copy of ci^{Cell}) are sufficient to supply this function.

In Vivo Evidence that the Repressor Activity of Ci, but Not that of Ci^{Cell}, Is Subject to Hh Regulation

It has been proposed that Ci repressor activity is negatively regulated by Hh signaling (Aza-Blanc et al., 1997). To analyze the influence of Hh signaling on Ci repressor activity in vivo, we used the P compartment of the wing imaginal disc as an assay system. Although P cells normally do not express Ci, they express *hh*, providing them simultaneously with Hh ligand and with a reporter gene to assay the repressor activity of Ci (*hh* itself). We controlled the ability of P cells to transduce the Hh signal by manipulating the function of *smoothened* (*smo*), which encodes the transducing component of the Hh receptor complex (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). P cells homozygous mutant for *smo* express *hh* at wild-type levels (Figure 2C). However, *smo* mutant P cells of discs that express Ci ubiquitously from a transgene fail to express *hh* (Figure 2D), indicating that Ci functions as a potent repressor of *hh* expression in the absence of the Hh signal transduction. Since the *hh-lacZ* gene is expressed in neighboring smo^{+} P cells that express the same *ci* transgene, it appears that reception of the Hh signal in these cells prevents Ci from repressing *hh* transcription. Thus, the ability of Ci to function as a transcriptional repressor is regulated by Hh. By contrast, Ci^{Cell} represses *hh* transcription in P cells regardless of the presence of *smo* activity (Figure 2E). We conclude that Ci^{Cell} escapes Hh regulation and acts as a constitutive repressor.

Ci Functions as an Activator, Rather Than a Repressor, of *ptc* and Late *engrailed* Expression

Anterior compartment cells along the A/P compartment boundary express high levels of *ptc* in response to Hh signaling, in contrast to cells in the remainder of the A compartment, which express only low levels of *ptc*. During a late stage of wing imaginal disc development, these "boundary" A compartment cells also express *en* (Blair, 1992). The upregulation of these target genes could result from the reduction of Ci repressor activity in response to Hh signaling. Clones of A compartment cells homozygous for the ci^{94} mutation do not appear to express high levels of *ptc* or to express *en* (Figures 3A–3C), regardless of where they arise, except for large clones in the notum that are associated with weak upregulation of a *ptc-lacZ* gene. Strikingly, clones located very close to the A/P compartment boundary abolished Ptc and En expression (Figures 3B and 3C). Since elimination of Ci leads to a loss, rather than a gain, of *ptc* and *en* expression, we conclude that Hh signaling normally upregulates the expression of these genes by creating an activating form of Ci rather than eliminating the expression or activity of a repressing form.

In Vivo Evidence that the Activator Activity of Ci Is Subject to Hh Regulation

To test whether the transcriptional activator function of Ci is created in response to Hh signaling, we again used P compartment cells as an assay system. Clones of *smo* mutant cells in the P compartment of wing discs that ubiquitously express *ci* do not express *ptc-lacZ* (Figure 3D), indicating that Ci is unable to induce *ptc-lacZ* expression in the absence of Hh input. By contrast, neighboring P cells that are smo^{+}, and hence able to receive the Hh signal, readily express *ptc-lacZ* (Figure 3D). The equivalent behavior has previously been described for A compartment cells that fail to upregulate *ptc* in the absence of *smo* function (Chen and Struhl, 1996). Thus, the activator activity of Ci is not constitutive, but rather depends on the reception of the Hh signal.

Activator Activity of Ci Is Controlled by a Mechanism Distinct from the Hh-Regulated Proteolysis

Full-length Ci (Ci-155) undergoes proteolytic cleavage to give rise to the N-terminal fragment Ci-75 (Aza-Blanc et al., 1997). We have shown that Hh signaling blocks the repressor activity of Ci (Figure 2D). Thus, Hh signaling might generate the activating form of Ci merely by stabilizing the intact protein that has an inherent activating function. To test this hypothesis, we sought to create

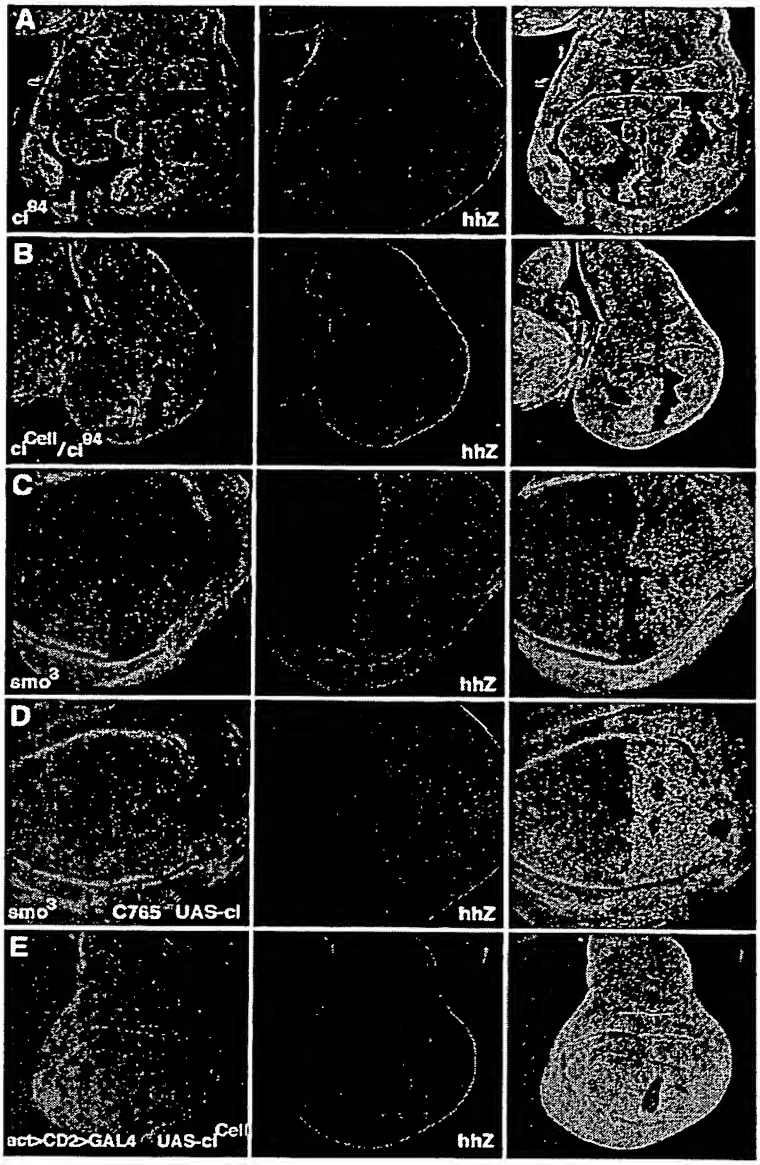

Figure 2. Activity of the Repressor Form of Ci, which Inhibits Expression of *hh* at Physiological Levels, Is Regulated by Hh Signaling

Clones of cells expressing no Ci (homozygous ci^{84} clones, [A]), only ci^{Cell} (ci^{Cell}/ci^{84} clones, [B]; or posterior *act5c>Gal4 UAS-*ci^{Cell} clones, [E]) or no functional Smo (homozygous smo^3 clones; [C andD]) are marked by the absence of GFP staining (A and B) or CD2 staining (C–E), in green (left column). *hh-lacZ* expression (hhZ) is shown in red (central column). The superimposition of both stainings is shown to the right. In this and subsequent figures, third instar wing imaginal discs are shown with anterior to the left and dorsal up. (A) Wing imaginal disc with several ci^{84}/ci^{84} clones. The absence of Ci in anterior clones relieves the repression of *hh*. The observed upregulation of *hh* is weak in the wing pouch, strong in the hinge, and often absent in the notum. (B) One copy of ci^{Cell} is sufficient to prevent ectopic *hh* expression in anterior ci^{Cell}/ci^{84} clones. (C and D) Formation of Ci[rep] is controlled by Hh. P cells are normally devoid of Ci and express *hh* irrespective of whether they are mutant for *smo* or not (C). However, P cells ectopically expressing Ci efficiently repress *hh* expression if they are mutant for *smo* but not if they are smo^+ (D). In (D), *UAS-ci* expression is driven by the ubiquitously active *Gal4* driver line *C765* (Nellen et al., 1996). (E) Ci^{Cell} escapes Hh control and functions as a constitutive inhibitor of *hh* expression. Posterior cells expressing ci^{Cell} that were generated by means of an *act5c> CD2>Gal4* UAS-ci^{Cell} *hsp70-flp* transgene combination and marked by the absence of CD2 staining inhibit *hh-lacZ* expression regardless of the state of smo^+ activity.

a form of Ci that is not cleaved in vivo. The Ci cleavage site has been mapped to a region encompassing amino acids 650 to 700 (Aza-Blanc et al., 1997). In our initial attempt to remove the cleavage site, we deleted sequences encoding amino acids 612 to 712 of the Ci protein (Figure 4A). However, Western blot analysis of this mutant protein (Ci^C) revealed that it could still be proteolyzed to Ci-75 (data not shown). We next deleted sequences encoding amino acids 611 to 760. This mutant protein, referred to as Ci^U (see Figure 4A), did not yield appreciable amounts of Ci-75 and therefore appears to be resistant to proteolysis (Figure 4D).

We expressed Ci, Ci^C, and Ci^U ubiquitously in wing discs in which we also induced *smo* mutant clones. As described above, *hh-lacZ* expression is abolished in P compartment clones of smo^- cells expressing Ci (see Figure 2D). The same result was obtained with Ci^C (data not shown). By contrast, Ci^U expression did not abolish *hh-lacZ* expression in smo^- P cells (Figure 4B), indicating that Ci^U is unable to provide repressor function even in the absence of Hh signaling. This result indicates that cleavage of Ci-155 to Ci-75 is a necessary step in the formation of Ci repressor.

Since Ci^U cannot undergo proteolysis to Ci-75, we then asked whether it functions as a constitutively active form of Ci. Ubiquitous expression in wing discs revealed that Ci^U, like Ci^C and Ci, readily activates *ptc-lacZ* in P compartment cells, but not in anterior A cells, nor in

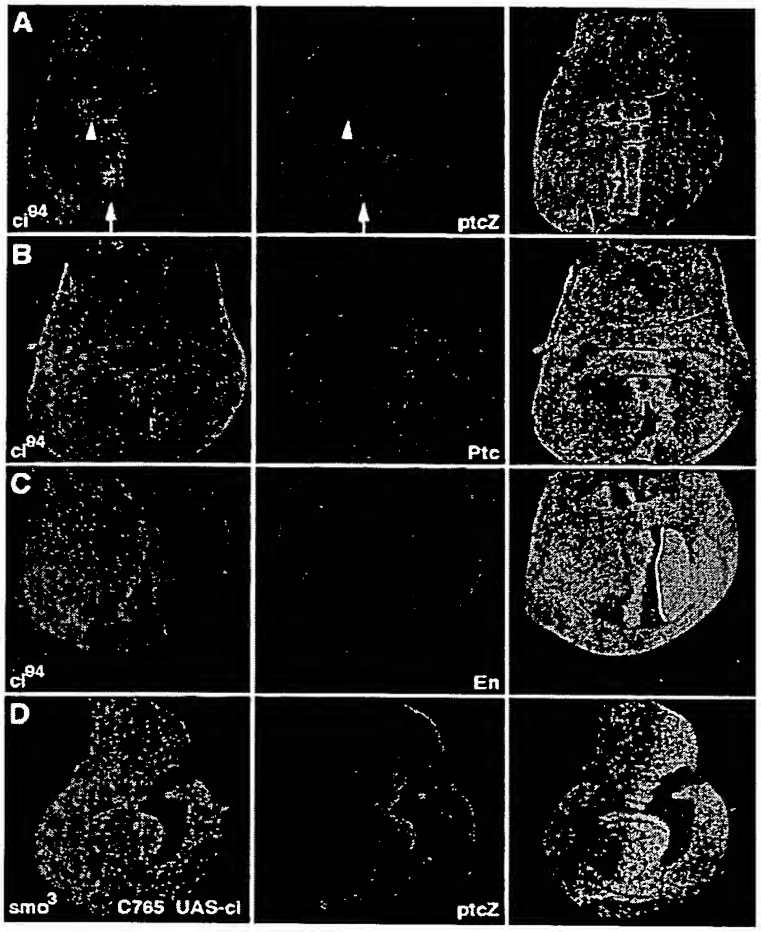

Figure 3. *ptc* and *en* Expression Are Controlled by the Activator Function of Ci, which in Turn is Subject to Hh Regulation

ci^{94} homozygous clones (A–C) and smo^3 clones (D) are marked by the absence of Ci staining (A) or absence of GFP staining (B–D) in green. *ptc-lacZ*, Ptc, and En expressions are shown in red. (A) Absence of Ci does not lead to *ptc* upregulation in the wing primordium (arrow). Nonautonomous *ptc-lacZ* (ptcZ) induction can be observed in hinge clones (arrowhead). (B) Ci is required for Ptc expression. No Ptc protein is made in anterior boundary cells that lack Ci. In large notum clones, Ptc protein is not affected by the absence of Ci. (C) Late anterior En expression (Blair, 1992) is abolished in ci^{94} clones abutting the compartment boundary and is not upregulated in clones located more anteriorly. The location of the compartment boundary is indicated in the panel on the right. (D) The activator function of Ci is controlled by Hh. *ptc-lacZ* upregulation in P cells that ubiquitously express Ci under the *Gal4* driver *C765* is abolished in posterior smo^3 clones.

smo mutant P compartment cells (Figure 4C). Thus, Ci^u functions as an activator form of Ci, but it does so only in Hh-receiving cells, indicating that the activating function of the Ci^u protein depends on Hh signal transduction. We conclude that the prevention of proteolysis to Ci-75 is not sufficient to cause Ci to function as an activator and that activator function requires an additional Hh-dependent step.

dpp Transcription Is Regulated by a Combination of Ci[act] and Ci[rep] Activities

We have provided evidence that Ci exhibits two activities in its control of target gene expression: an activator activity (named Ci[act]) and a repressor activity (Ci[rep]), both of which are regulated by Hh. Our results also indicate that for some genes, Ci acts only as an activator of transcription (*ptc*, *en*), and for others, exclusively as an inhibitor (*hh*). The most critical target gene of Hh in its control of wing development is *dpp*. To determine which of Ci's activities control *dpp* expression, we examined the expression of *dpp-lacZ* reporter genes in wing discs carrying *ci* mutant clones. As shown in Figure 5A, ci^{94} mutant cells located in the A compartment invariably express *dpp-lacZ*. However, the level of expression was less than that observed in wild-type cells located along the A/P boundary. About 30% of the clones also caused nonautonomous expression of *dpp-lacZ* in neighboring cells at higher levels than inside the mutant clones (Figure 5A). To determine whether *dpp-lacZ* expression observed both within and surrounding clones of ci^{94} mutant cells is due to ectopic Hh expressed by the mutant cells (see Figure 2A), we generated ci^{94} hh^- double mutant clones. All clones analyzed ($n > 50$) exhibited autonomous *dpp-lacZ* expression at levels lower than the endogenous *dpp-lacZ* stripe. Nonautonomous *dpp-lacZ* expression was never observed (Figure 5B). These results show that *dpp* expression within ci^- clones is Hh independent, but nonautonomous *dpp* expression around the *ci* mutant clones is Hh dependent. Thus, one function of Ci in the absence of Hh signaling is to repress a low, latent transcription of *dpp* in A cells. Consistent with this interpretation, we found that a single copy of the ci^{Ceh} allele (in ci^{Ceh}/ci^{94} clones) completely suppressed ectopic *dpp* expression in the A compartment as well as normal *dpp* expression in cells near the

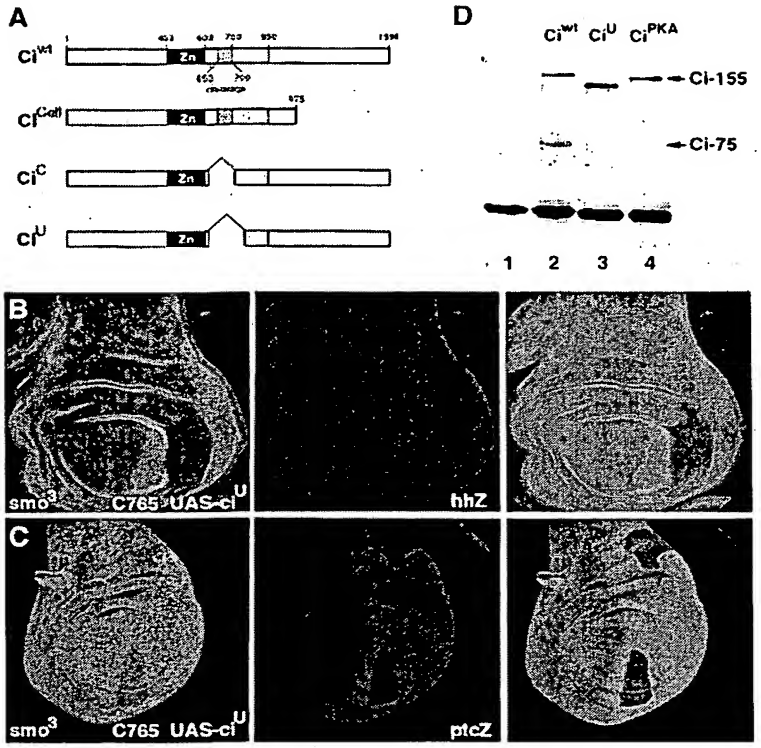

Figure 4. Ci^{U}, an Uncleavable Form of Ci, Lacks Repressor Activity but Is Still Subject to Hh Control

(A) Schematic representation of Ci protein and mutant derivatives used in this study. Zinc fingers (black box): amino acids 453 to 603; cleavage region (stippled box): residues 650 to 700; tethering domain (gray box): amino acids 700 to 850 (Aza-Blanc et al., 1997). *ci^{Cell}* encodes a protein truncated at amino acid 975. In Ci^{C}, amino acids 612 to 712 are absent, and in Ci^{U}, residues 611–760.

(B) Ci^{U} is unable to generate Ci[rep], even in the absence of Hh signaling. In posterior cells expressing Ci^{U} (*C765-Gal4* driving *UAS-ci^{U}*), *hh-lacZ* expression (red) is unaffected by the loss of *smo* (absence of anti-CD2 staining, green).

(C) Ci^{U} requires Hh input for activity because *ptc-lacZ* expression (red) induced by ubiquitous expression of Ci^{U} is abolished in *smo* mutant P cells (marked by absence of green anti-GFP staining).

(D) Ci^{U} does not give rise to Ci-75. Kc167 cells were transfected with various HA-tagged Ci constructs. Immunoprecipitation followed by Western blotting shows that wild-type Ci is partially cleaved into Ci-75 (lane 2). Very little, if any, Ci-75 is generated from Ci^{U} (lane 3) or from Ci mutated at a putative PKA phosphorylation site (Ci^{PKA}; lane 4) that had been shown previously to be resistant to cleavage (Chen et al., 1998). Lane 1, untransfected cells.

A/P boundary (Figure 5C). However, our observation that the levels of *dpp* expression in *ci* null clones are significantly lower than those in cells receiving the Hh signal indicates that Ci is also required to activate high levels of *dpp* transcription. Lack of Ci is not sufficient to induce maximal *dpp* expression. This can best be seen in *ci^{94}* clones located within the *dpp-lacZ* stripe along the A/P boundary where the low *dpp* expression levels of mutant cells contrast with those of their neighboring wild-type cells (Figure 5D). Thus, our results indicate that *dpp* transcription in the A compartment is regulated by a combination of repressor and activator activities of Ci.

The requirement for both Ci[rep] and Ci[act] to generate the proper *dpp* expression domain is further illustrated in wing imaginal discs containing low amounts of ubiquitously expressed Ci. Such discs were obtained by rescuing animals homozygous for *ci^{94}* with a transgene driving *ci* expression under the control of the weak *tubulinα1* promoter (*tub-ci*). This transgene rescues the *ci^{94}* mutation to the pupal stage, with rare adult escapers. Three different insertions of the *tub-ci* transgene were analyzed. Wing imaginal discs from *ci^{94}* mutant larvae rescued with a *tub-ci* transgene that produces relatively robust levels of Ci (Figure 6A) showed a nearly normal *dpp* expression domain (Figure 6C). Discs with medium amounts of Ci exhibited ubiquitous, weak *dpp* expression, with a slightly higher level near the A/P boundary (Figure 6D). These discs did not show detectable *hh-lacZ* expression in A cells (not shown), indicating that sufficient amounts of Ci[rep] may nevertheless be present to prevent *hh* expression. Thus, lower amounts of Ci[rep] are required for the repression of *hh* expression in A cells compared to *dpp*. Finally, discs with very low amounts of Ci exhibited a grossly distorted morphology, expressed *dpp* ubiquitously in the A compartment, and did not show an increase of *dpp* expression at the A/P boundary (Figure 6E). Such discs also expressed *hh-lacZ* ubiquitously at low levels in A cells (Figure 6F) but not *ptc-lacZ* (not shown). These experiments indicate the requirement for a sufficient amount of Ci repressor to inhibit inappropriate expression of both *dpp* and *hh* away from the A/P boundary and for a sufficient amount of the Ci activator to fully induce *dpp* transcription in response to Hh signaling along the A/P boundary.

The consequences of lack of Ci repressor cannot be overcome by proper amounts of regulated activator, as can be seen when the Ci^{U} protein is expressed in place of wild-type Ci (i.e., in animals homozygous for *ci^{94}* "rescued" by a *ci^{+}* genomic transgene altered to encode Ci^{U}). Although such animals survive to early pupal stages, their discs exhibit grossly enlarged A compartments (Figure 6G). All A cells ectopically express *dpp* (Figure 6H) and, to a lower extent, also *hh* (Figure 6I), indicating that the generation of Ci[rep] is essential to repress *dpp* and *hh* in these cells. By contrast, the *ptc-lacZ* expression pattern was virtually normal (Figure 6J), and *dpp-lacZ* expression was elevated in cells close to the A/P boundary, confirming that these two aspects of gene regulation only require the activator function of

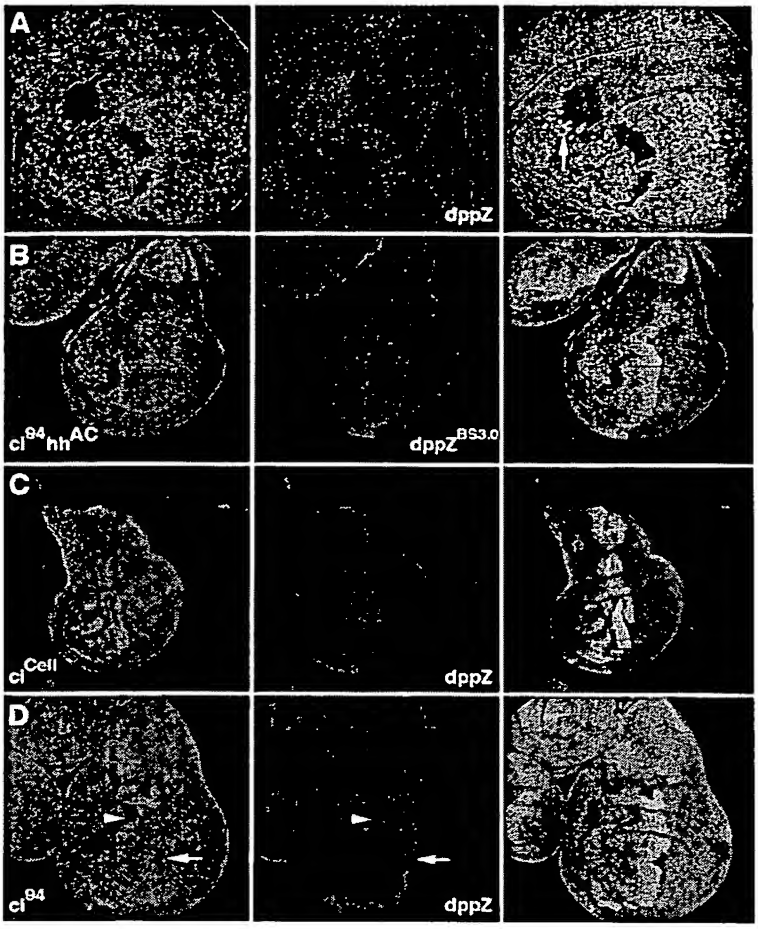

Figure 5. *dpp* Expression Is Controlled by Both Activator and Repressor Functions of Ci

Clones are marked by the absence of GFP staining (green). *dpp* expression is visualized in red by the *dpp-lacZ*^{P10638} reporter (dppZ, [A], [C], and [D]) or by the *dpp-lacZ*$^{BS3.0}$ transgene ([B]). (A) *ci*^{94} homozygous clones. *dpp* is ectopically expressed in clones outside the normal *dpp* expression domain, but at levels lower than those within the endogenous stripe. In some instances ([A], see arrow), *dpp* expression is activated nonautonomously at levels higher than those inside the clone. (B) *ci*^{94} *hh*$^{-}$ double mutant clones. The autonomous expression of *dpp* is unaffected, while nonautonomous *dpp* induction is abolished. (C) *ci*^{Cell}/*ci*^{94} clones. No upregulation of *dpp* is observed. Clones that fall within the endogenous *dpp* stripe fail to express *dpp*. (D) *ci*^{94} clones positioned within the stripe of endogenous *dpp* expression exhibit a reduction in *dpp* expression (arrow). The levels are comparable to those of more anteriorly located *ci*^{94} clones (arrowhead).

Ci, which is present in Ci^{U} and appears to be properly regulated by Hh.

En Represses *dpp* Expression in P Compartment Cells Directly and through the Repression of *ci* Transcription

It has been proposed that the activities of En and its closely related homolog Invected (Inv) prevent *dpp* expression in P cells by binding to regulatory sequences in the *dpp* enhancer (Sanicola et al., 1995). Indeed, P compartment clones double mutant for *en* and *inv* express high levels of *dpp* (Figure 7A; Sanicola et al., 1995; Zecca et al., 1995). However, since En/Inv negatively regulate *ci* expression (Eaton and Kornberg, 1990), *dpp* expression in such P compartment clones might reflect a Ci-mediated response to Hh secreted by surrounding cells. Adding to these uncertainties, experimental overexpression of *ci* in P cells can lead to *dpp* expression in the presence of En/Inv (Dominguez et al., 1996; Hepker et al., 1997). To assess the contribution of En and Inv to *dpp* repression in P compartment cells, we removed *en/inv* function together with other genes involved in *dpp* regulation. First we compared the properties of posterior *en/inv* double mutant clones (Figure 7A) with those of clones that in addition lack *smo* function (Figure 7B). In *en/inv smo* triple mutant clones, *dpp-lacZ* expression is abolished. Hence, in the absence of the Hh signal, the loss of En/Inv function does not cause *dpp* expression. By contrast, cells that are homozygous mutant for *en/inv* and in addition for *ci*^{94} (*en/inv ci*^{94}) retain *dpp* expression at levels that are lower than those of anterior wild-type boundary cells but are equal to those of mutant anterior cells (Figure 7C). The expression of *dpp* in posterior *en/inv ci*^{94} triple mutant cells is not dependent on Hh input, as the same behavior is observed in quadruple mutant cells that, in addition to *en/inv* and *ci*, also lack *smo* function (*en/inv ci*^{94} *smo*^{3} clones, Figure 7D). Thus, in the coincident absence of En/Inv and Ci, *dpp* is expressed at a basal level in P cells. The simplest interpretation of these results is that in *en/inv* mutant clones, Ci protein is made but does not repress *dpp* transcription, due to the reception of Hh emanating from surrounding cells. Instead, Ci functions as a Hh-dependent activator for *dpp* and increases *dpp* transcription beyond the basal level. In *en/inv smo* mutant clones, no Hh signal is transmitted, and Ci assumes repressor function to prevent the basal activity of the

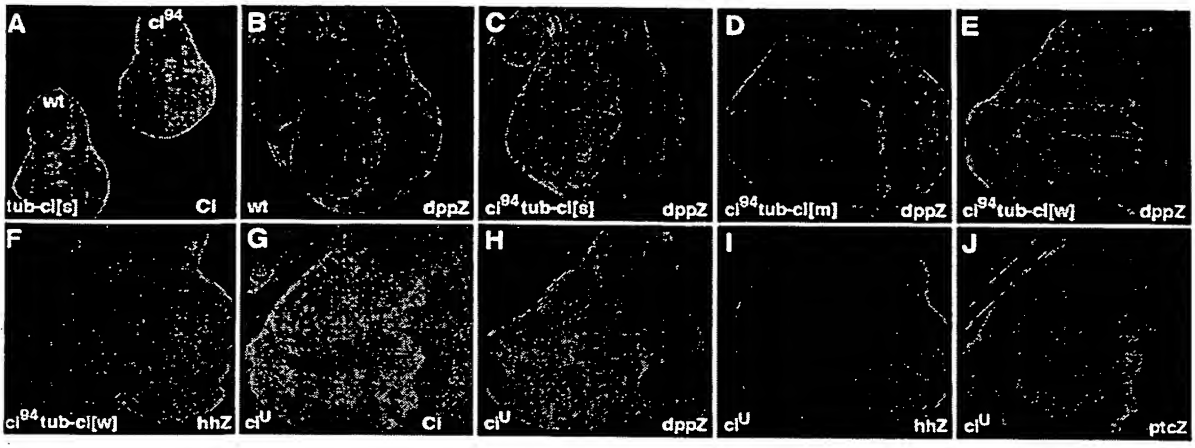

Figure 6. Insufficient Ci[rep] Levels Result in Ectopic *dpp* and *hh* Expression

ci^{94} homozygous discs expressing *ci* under the control of the *tubulinα 1* promoter (*tub-ci*, [C–F]) or expressing Ci^{U} (*P[ci^U]*, [G–J]). Lines expressing different amounts of Ci were tested. Ci protein staining is shown in green and reporter gene activity (*hh-lacZ*, *dpp-lacZ*, *ptc-lacZ*) in red. All discs are shown at the same magnification except in [A], where a 2-fold lower magnification was used. (A) Comparative Ci protein staining of two imaginal discs from sibling larvae that are genotypically wild-type (wt) for *ci* or homozygous null mutant (ci^{94}), respectively. Both discs contain one copy of the strongly active *tub-ci* transgene, designated *tub-ci[s]*, and are oriented with the anterior side to the left. (B–E) *dpp-lacZ* expression pattern in a wild-type disc (B) compared to ci^{94}/ci^{94} mutant discs expressing a strong (C), a medium (D), and a weak (E) *tub-ci* transgene. The three insertions of the *tub-ci* transgene were selected on the basis of differing anti-Ci staining intensities. (F) Ectopic *hh* expression in ci^{94} discs rescued by the weak *tub-ci* transgene. (G) Ci^{U} protein expression pattern in homozygous ci^{94} discs expressing a single copy of the genomic *P[ci^U]* transgene. (H) *dpp-lacZ* expression in homozygous ci^{94} disc containing two copies of the *P[ci^U]* transgene. Weak *dpp-lacZ* expression is present throughout the anterior compartment, with an upregulation near at the compartment boundary. (I) Ectopic *hh-lacZ* expression is observed in homozygous ci^{94} discs containing one copy of the *P[ci^U]* transgene. (J) *ptc-lacZ* expression is only mildly affected in homozygous ci^{94} discs containing one copy of the *P[ci^U]* transgene. Some anterior cells along the D/V boundary express *ptc-lacZ*, likely in response to ectopic Hh.

dpp gene. The thin stripe of *dpp* expression in wild-type discs reflects the composite control of basal *dpp* transcription by En and Ci, the latter regulated by Hh. In the P compartment, En blocks basal *dpp* transcription both directly and by repressing *ci* transcription that would otherwise yield the Ci[act] function. In the A compartment, Ci[rep] represses basal *dpp* expression in the absence of Hh signaling, while in its presence, expression of the Ci[rep] function is replaced by Ci[act], yielding high levels of *dpp* transcription in cells along the compartment boundary.

Discussion

Hh proteins control a multitude of fundamental patterning processes during animal development. Here, we are concerned with the mechanism by which Hh regulates the expression of target genes, by asking how Hh signaling controls the activity of the transcriptional regulator Ci. Our results establish discrete activator and repressor roles of Ci in *Drosophila* limb development and demonstrate that the expression of different Hh target genes requires distinct contributions of these forms of Ci. Our results also indicate that the state of Ci activity is controlled by a Hh-dependent mechanism other than, or in addition to, Ci cleavage.

Evidence for a Regulated Activator Activity of Ci

Previous studies provided conflicting results regarding the nature of Ci's transcriptional regulatory functions and their control by Hh. The recent discovery that full-length Ci undergoes limited proteolysis to form Ci-75, which translocates to the nucleus, focused the attention on the regulated generation of Ci-75 (Aza-Blanc et al., 1997). The formation of Ci-75 is inhibited by Hh in vitro, and Ci-75 can repress the transcription of *hh* and *ptc* when overexpressed in vivo (Aza-Blanc et al., 1997). Our demonstration that Ci functions as a repressor of *hh* and *dpp* transcription at physiological concentrations, that this activity is controlled by Hh in vivo, and that it depends on the ability of Ci to be cleaved provides strong support for the view that Hh indeed exerts some aspects of gene regulation through the inhibition of Ci-75 formation. However, the ability to entirely remove *ci* gene function and the use of a form of Ci that cannot be cleaved (Ci^{U}) allowed us to identify Hh target genes (*ptc* and *en*) whose expressions do not depend on Ci repressor formation. Because the expression of these genes depends both on Hh signaling and on *ci* function, we interpret our results as evidence that Ci must also function as a transcriptional activator in vivo and that this activity must be regulated by Hh.

Essential In Vivo Roles for Both Regulatory Activities of Ci

The existence of two opposing transcriptional activities of Ci raises the question as to what extent each of them is required for Hh-mediated patterning. Key for us in addressing this issue was our approach of completely removing *ci* function and replacing it with altered forms

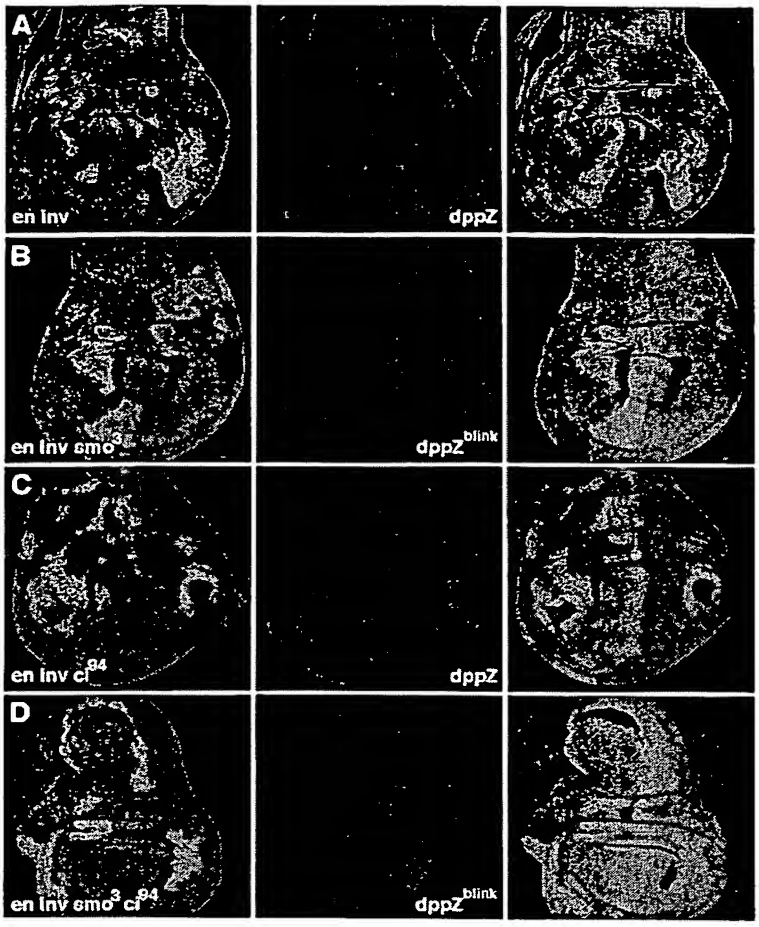

Figure 7. En and Ci[rep] Restrict the *dpp* Expression Domain to Anterior Boundary Cells

(A) *en-inv* double mutant clones autonomously express *dpp* in the P compartment. The *dpp-lacZ* expression level is similar to that found in anterior boundary cells.

(B) *en inv smo*^{3} triple mutant clones show no ectopic *dpp* expression in P cells. The *dpp* reporter gene here is *dpp*^{blink}*-lacZ*.

(C) *en inv ci*^{94} triple mutant clones in the P compartment exhibit autonomous, ectopic *dpp-lacZ* expression. In contrast to *en inv* clones, the *dpp* expression level is lower than that observed in wild-type anterior boundary cells but is similar to the level observed within anterior clones.

(D) *en inv smo*^{3} *ci*^{94} quadruple mutant clones express *dpp*^{blink}*-lacZ*.

of *ci* encoding derivatives that lack either activator function (Ci^{Cell}) or repressor function (Ci^{U}). Animals expressing Ci^{U} instead of wild-type Ci suffer during imaginal development from a failure to repress *dpp* and *hh* expression. Other aspects of Ci function, such as the regulation of *ptc* and *en* transcription by Hh, occur normally. Although Ci^{U} activity is unable to rescue *ci*^{94} homozygous animals to adulthood, it does support development of some *ci*^{Cell}/*ci*^{94} animals to adult stages (N.M. and K.B., unpublished results). This suggests that activator (here Ci^{U}) and repressor (Ci^{Cell}) functions of Ci can be provided by separate genes, a design that appears to have been adopted by the vertebrate Gli proteins (Marigo et al., 1996). Interestingly, *ci*^{U} animals survive embryogenesis, indicating that Hh target genes are not misregulated during embryonic stages. Although we cannot formally exclude the possibility that during embryogenesis a fraction of Ci^{U} is cleaved into some form of Ci[rep], we favor the explanation that during embryonic development Ci function does not depend on repressor activity but functions primarily by activating transcription of Hh target genes, such as *wg*. This is consistent with the phenotype of *ci* null mutant embryos (Slusarski et al., 1995) that resemble *wg* mutant embryos, as opposed to embryos in which Wg or Hh signaling occurs ectopically. Thus, the Ci repressor branch of Hh signaling appears to operate only during adult development, where it is essential to suppress basal transcription of the *dpp* and *hh* genes.

Our results with *ci*^{U} and *tub-ci* transgenes also clarify how *hh* expression is repressed in anterior boundary cells. These cells are exposed to Hh levels that prevent Ci[rep] formation most effectively and would thus be expected to express *hh*. The absence of *hh* transcription in these cells could be explained by the Ci[act]-dependent induction of a *hh*-specific transcriptional repressor. Yet *ci*^{U} animals ectopically express *hh* in anterior boundary cells despite their normal regulation of Ci[act] activity, indicating that Ci[rep], and not Ci[act], prevents ectopic *hh* expression in wild type. Thus, we conclude that sufficient amounts of Ci[rep] are present in Hh-receiving anterior boundary cells to repress *hh* transcription.

The absence of Ci activator function is also fatal, as observed in embryos homozygous mutant for Ci^{Cell} (Orenic et al., 1990). However, Ci^{Cell} is not regulated by Hh and hence does not serve as an unambiguous argument that Ci activator activity is required in addition to regulated repressor activity. Nonetheless, the dependence of embryonic *wg* expression on Ci activator function and the role of Ptc in restricting the range of Hh action

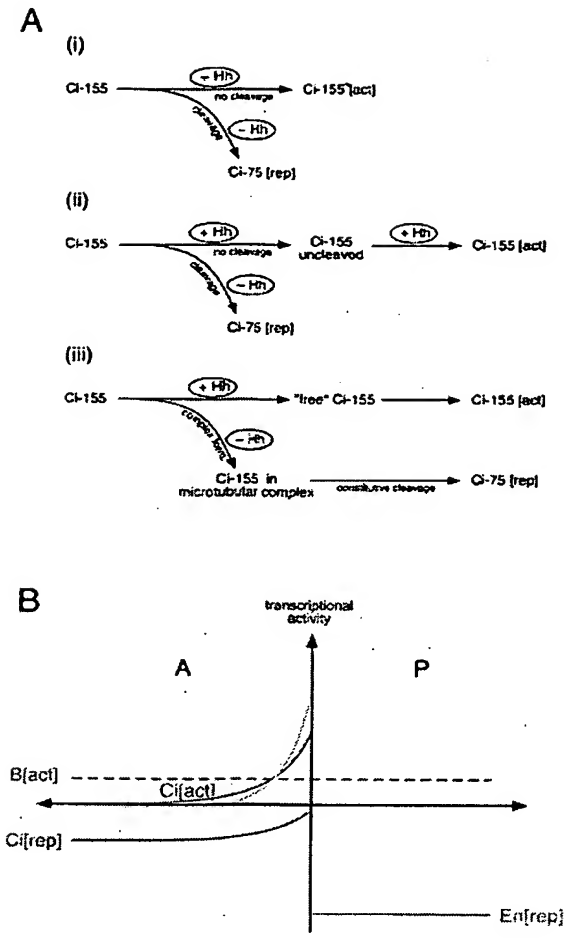

Figure 8. Models Illustrating how Ci[rep] and Ci[act] Are Controlled by Hh and how These Transcriptional Activities of Ci Shape the Dpp Morphogen Source

(A) Three models that depict the point at which Hh might exert its effect in the signaling cascade. In (i), Hh controls the signaling output by directly affecting Ci proteolysis. Prevention of Ci[rep] formation leads to more Ci[act]. Such a model would predict that an uncleavable form of Ci is constitutively active. To accommodate the fact that Ci^{u} is not constitutively active, models (ii) and (iii) are proposed. In (ii), presence of Hh spares Ci-155 from proteolytic processing. Protected, full-length Ci must undergo one or more Hh-dependent activation steps to mature into a transcriptional activator. These steps could include posttranslational modifications, release of Ci-155 from a cytoplasmic tethering complex, or nuclear translocation. In (iii), Hh does not act on cleavage, but rather on the subcellular localization of Ci. Absence of Hh leads to the incorporation of Ci into a microtubule-associated complex, where Ci is processed by default into Ci[rep]. Presence of Hh would prevent microtubular complex formation and thus, indirectly, also cleavage. Although here a single Hh-dependent control point would satisfy our data, we cannot exclude that other maturation steps, such as those evoked in (ii), are important. "Free" Ci-155 refers to either uncomplexed Ci or precomplexed Ci.

(B) The concerted action of Ci[act], Ci[rep], and En shape the Dpp morphogen source. Shown here are the effects of Ci[act] (green), Ci[rep] (red), and En (orange) on the *dpp* transcription output (yellow). Since Ci[rep] and En both exhibit repressive activities, they are depicted on the negative side of the vertical scale. In the absence of Ci and En, *dpp* is expressed ubiquitously in the wing imaginal disc (B[act] for the activity of the basal enhancer B, blue stippled line). In the P compartment, En represses the *dpp* basal expression directly. In anterior regions of the A compartment, no Ci[act] is present, and the negative effect of Ci[rep] on basal *dpp* transcription is sufficient to shut it off completely. Near the compartment boundary, diffusion of Hh from P to A cells decreases the levels of Ci[rep] and increases Ci[act] activity. The total *dpp* transcription output (in yellow) is the sum of the contributions of Ci[act], Ci[rep], and B[act] at each point along the A–P axis of the A compartment. The combined activities of Ci[act] and Ci[rep] result in a more intense, but less extended, domain of *dpp* transcription.

(Chen and Struhl, 1996) allow us to attribute an essential in vivo role to the Hh-regulated activator activity of Ci.

Regulation of Ci by Hh: Evidence for a Control Step Other Than Proteolytic Cleavage

One key finding of our results is that Hh controls at least two aspects of Ci function. Apart from negatively regulating the generation of a repressor form of Ci, Hh signaling tightly controls the formation of an activator form of Ci. The simplest model to account for both steps of Hh regulation would be that the formation of Ci[act] is a direct consequence of preventing the formation of Ci[rep] and vice versa (Figure $8A_i$). However, the mere prevention of Ci-75 formation does not render the full-length Ci protein active by default. This argument is based on our observation that an uncleavable form of Ci, Ci^{u}, lacks activator activity in the absence of Hh input. Thus, our results suggest the existence of an additional step of Hh regulation that converts Ci into a transcriptional activator. We present below two models that can account for both outputs of Hh signaling.

As illustrated in the model of Figure $8A_{ii}$, two separate steps of Hh regulation could be required for the generation of Ci[act], the first one being the sparing of Ci-155 from degradation to Ci-75, and the second one being an activation step for nonprocessed Ci to acquire activator activity. One component of the Hh signaling cascade that could be responsible for the Hh-dependent activating step of Ci is the Ser/Thr kinase Fused (Fu). Such a role for Fu has recently been proposed (Ohlmeyer and Kalderon, 1998). In wing imaginal discs, however, Fu kinase function does not appear essential for many aspects of Hh signaling. Although anterior *fu* mutant cells fail to express late *en* (Sanchez-Herrero et al., 1996), they still transcribe *ptc* (albeit at lower levels than wild-type cells) and exhibit nearly normal levels of *dpp* expression (Alves et al., 1998). Thus, Fu activity can not fully account for the potent activation of *ptc* and *dpp* expression by Ci in response to Hh. Moreover, in the absence of Su(fu), Fu kinase activity is dispensable (Préat et al., 1993). Because *fu Su(fu)* double mutant animals develop virtually like wild-type animals, it can be inferred that the Ci activator function is regulated properly by Hh in the absence of Fu. Together, these observations suggest that while Fu activity may be involved in some second-order level of regulation permitting the induction of less sensitive Hh targets by Ci (such as late *en*), the primary mechanism by which Hh

signaling regulates the formation of Ci[act] does not depend on the catalytic function of Fu.

Another model to explain our experimental results is based on the premise that Hh signaling governs the fate of Ci primarily by controlling its cytoplasmic association with multiprotein complexes containing the kinesin-related protein Cos2 (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; see also Ingham, 1998). If complex formation is a prerequisite for targeting Ci to its site of proteolytic processing, the main function of Hh signaling might be to prevent this association and thereby indirectly also spare Ci-155 from being processed by the proteolytic cleavage machinery (see model, Figure 8A$_{iii}$). A prediction of this model would be that a cleavage-resistant form of Ci would still be incorporated into the microtubule-associated complex and thus still be subjected to Hh control. Ci^{U} fulfills this criterion.

The Combination of Three Transcriptional Activities Shapes the *dpp* Expression Domain

The function of Hh in controlling growth and pattern of the wing primordium is mediated to a large extent by the local expression of Dpp that is secreted from a subset of anterior cells in response to Hh signaling. Dpp acts directly, at long range, and in a concentration-dependent manner to convey positional information to wing cells along the anteroposterior axis (Lecuit et al., 1996; Nellen et al., 1996). Thus, the precise domain in which *dpp* is expressed and the absolute levels of Dpp secreted are consequential for the morphogenesis of the wing (e.g., Zecca et al., 1995). It may not be coincidence, therefore, that it is precisely the *dpp* gene that is subject to both modes of Ci control. In the simultaneous absence of *ci* and *en* function, *dpp* is expressed at a constitutive basal level in all wing cells. From this we surmise the existence of a ubiquitous enhancer (B, for basal) that stimulates *dpp* transcription by default. Our results indicate further that both regulatory inputs, Ci[act] and Ci[rep], act on *dpp*, and we propose that their superimposition serves to "sharpen" the Dpp morphogen source (Figure 8B). Two consequences can be invoked from the combination of the two regulatory mechanisms: first, a narrowing of the *dpp* stripe, and second, an increase in *dpp* expression levels (Figure 8B). Finally, we note that the dual control of *dpp* expression by Ci necessitates a mechanism to prevent *dpp* transcription in P cells that contain neither form of Ci and would thus express *dpp* by default. This complication appears to be solved by subjecting *dpp* expression to repression by En. The result of all these regulatory measures is an exquisitely controlled system in which Dpp is secreted at high levels by a narrow strip of cells located along the A/P compartment boundary in the center of the wing primordium.

Experimental Procedures

Transgenes

Transgenes based on a *ci* cDNA (*tub-ci*, *tub-ci^{U}*, *tub-ci^{PKA}*, *UAS-ci*, *UAS-ci^{Cell}*, *UAS-ci^{F}*, *UAS-ci^{1}*) were made with cDNA clone *pJA-ci* (gift from J. Alcedo and M. Noll) that we altered to encode a triple HA-epitope tag at the N terminus of Ci. For Ci^{C} and Ci^{U}, Ci amino acids 612–712 and 611–760 were removed, respectively. For Ci^{PKA}, amino acids 837, 838, and 839 (DSQ) were mutated into alanine residues. For Ci^{Cell}, an NruI–SpeI fragment was replaced with the corresponding fragment derived from amplified *ci^{Cell}* genomic DNA. These constructs were sequenced to confirm that no other mutations were introduced. The DNA constructs encoding HA-tagged Ci, Ci^{C}, Ci^{U}, and Ci^{Cell} were inserted into pUAST (Brand and Perrimon, 1993) or into a P element plasmid containing the promoter of the *tubulinα1* gene (Basler and Struhl, 1994).

Transgenes based on the genomic *ci* gene (*P[ci^{+}]* and *P[ci^{U}]*) are derived from a 16 kb NotI fragment from λclone 6 (Locke et al., 1996) inserted into *pCaSpeR-4*. *P[ci^{U}]* was generated by exchanging an NcoI–NruI fragment of *P[ci^{+}]* with that of the Ci^{U} coding region described above.

Reporter genes used in this study were *dpp-lacZ$^{BS3.0}$* (Blackman et al., 1991), *dpp^{P1063}* (Zecca et al., 1995), *dpp-lacZ^{blink}* (a fragment from the *dpp^{blink}* enhancer; B. Müller and K. B., unpublished results), *hh^{P30}* (Lee et al., 1992), and *ptc(10.8L)A* (Chen and Struhl, 1996).

Additional transgenes used were *Gal4* driver *C765* (Nellen et al., 1996), *act5c>CD2>Gal4* (Pignoni and Zipursky, 1997), *P[smo^{+},hsp70-GFP]* (D. Nellen and K. B., unpublished results), and *hsp70-CD2* (Chen and Struhl, 1996).

Immunoprecipitation and Western Blots

The detection of the Ci cleavage product was essentially performed as described in Chen et al. (1998). Kc167 cells (10×10^{6} cells) were transfected by the calcium-phosphate method with 10 μg of *tub-ci*, *tub-ci^{U}*, or *tub-ci^{PKA}*, collected 3 days posttransfection, and lysed in buffer containing 50 mM Hepes (pH 7.4), 0.2 mM EDTA, 10 μM NaF, 250 mM NaCl, 1 mM DTT, 0.5% NP-40, 0.1% SDS, 1 mM PMSF, and protease inhibitors (Complete; Boehringer Mannheim). Extracts were precleared with protein G–agarose (Boehringer Mannheim). HA-tagged proteins were immunoprecipitated with rat monoclonal anti-HA (Boehringer Mannheim) and detected by Western blotting using mouse monoclonal anti-HA-11 (Babco). Proteins were visualized by chemiluminescence (ECL, Amersham).

Marked Clones of Mutant Cells

Larvae homozygous for the *ci^{94}* mutation and rescued by a *P[ci^{+}]*, *P[ci^{U}]*, or a *tub-ci* transgene were generated using parents that carry the *ci^{94}* or *ci^{Cell}* mutation in conjunction with the *Dp(1;4)1021[y^{+}]* chromosome. This allowed the unambiguous identification of homozygous mutant animals by their yellow mutant phenotype. Clones of mutant cells were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993) resulting in the loss of the *P[ci^{+}]* transgene in conjunction with an *hsp70-GFP* marker gene.

Genotypes of the larvae were as follows:

- ci^{94} clones, hhZ:
  y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; hh-lacZ/+; ci^{94}/ci^{94}
- ci^{Cell}/ci^{94} clones, hhZ:
  y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; hh-lacZ/+; ci^{Cell}/ci^{94}
- smo^{3} clones, hhZ:
  y w hsp70-flp; smo^{3} FRT39/hsp70-CD2 FRT39; hh-lacZ/+
- smo^{3} clones, C765 UAS-Ci, hhZ:
  y w hsp70-flp; smo^{3} FRT39/hsp70-CD3 FRT39; UAS-ci/C765 hh-lacZ
- UAS-ci^{Cell} clones, hhZ:
  y w hsp70-flp; act5c>CD2>Gal4/UAS-ci^{Cell}; hh-lacZ/+
- ci^{94} clones, ptcZ:
  y w hsp70-flp; ptc-lacZ/+; FRT82 P[ci^{+}] hsp70-GFP/FRT82; ci^{94}/ci^{94}
- ci^{94} clones, anti-Ptc, or anti-En:
  y w hsp70-flp; FRT42 P[ci^{+}] hsp70-GFP/FRT42; ci^{94}/ci^{94}
- smo^{3} clones, C765 UAS-ci, ptcZ:
  y w hsp70-flp; smo^{3} FRT40 ptc-lacZ P[ci^{+}] hsp70-GFP/FRT40; UAS-ci/C765
- smo^{3} clones, C765 UAS-ci^{U}, hhZ:
  y w hsp70-flp; smo^{3} FRT39/hsp70-CD2 FRT39; UAS-ci^{U}/C765 hh-lacZ
- smo^{3} clones, C765 UAS-ci^{U}, ptcZ:
  y w hsp70-flp; smo^{3} FRT40 ptc-lacZ P[ci^{+}] hsp70-GFP/FRT40; UAS-ci^{U}/C765

- ci^{94} clones, dppZ:
  y w hsp70-flp; dpp^{P1063B} FRT42 P[ci^+] hsp70-GFP/FRT42; ci^{94}/ci^{94}
- ci^{94} hh^{AC} clones, dppZ:
  y w hsp70-flp; dpp-lacZ$^{BS3.0}$/+; FRT82 P[ci^+] hsp70-GFP/FRT82 hh^{AC}
- ci^{94}/ci^{Ce6} clones, dppZ:
  y w hsp70-flp; dpp^{P1063B} FRT42 P[ci^+] hsp70-GFP/FRT42; ci^{Ce6}/ci^{94}
- en/inv clones, dppZ:
  y w hsp70-flp; dpp^{P1063B} FRT42 P[ci^+] hsp70-GFP/FRT42 Df(2R)en^E; ci^{94}/Dp(1;4)1021[y^+]
- en/inv smo^3 clones, dppZ:
  y w hsp70-flp; smo^3 FRT42 P[ci^+] P[smo^+,hsp70-GFP]/smo^3 FRT42 Df(2R)en^E; dpp-lacZ^{blink}/+; ci^{94}/Dp(1;4)1021[y^+]
- en/inv ci^{94} clones, dppZ:
  y w hsp70-flp; dpp^{P1063B} FRT42 P[ci^+] hsp70-GFP/FRT42 Df(2R)en^E; ci^{94}/ci^{94}
- en/inv smo^3 ci^{94} clones, dppZ:
  y w hsp70-flp; smo^3 FRT42 P[ci^+] P[smo^+,hsp70-GFP]/smo^3 FRT42 Df(2R)en^E; dpp-lacZ^{blink}/+; ci^{94}/ci^{94}

Immunochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained with appropriate antibodies to mark clones and monitor reporter gene expression. Antibodies were rat monoclonal anti-Ci 2A1 (gift from B. Holmgren), mouse monoclonal anti-Ptc (gift from I. Guerrero), mouse monoclonal anti-Engrailed 4D9 (gift from N. Patel), mouse monoclonal and rabbit polyclonal anti-GFP (Clonetech), mouse monoclonal anti-HA 12C5 (Boehringer), mouse monoclonal anti-CD2 OX34 (Serotec), and rabbit polyclonal anti-βGal (Cappel).

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References

Ahmed, A., and Podemski, L. (1997). Use of ordered deletions in genome sequencing. Gene *197*, 367–373.

Alcedo, J., and Noll, M. (1997). Hedgehog and its patched-smoothened receptor complex: a novel signaling mechanism at the cell surface. Biol. Chem. *378*, 583–590.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J.E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell *86*, 221–232.

Alexandre, C., Jacinto, A., and Ingham, P.W. (1996). Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. Genes Dev. *10*, 2003–2013.

Alves, G., Limbourg-Bouchon, B., Tricoire, H., Brissard-Zahraoui, J., Lamour-Isnard, C., and Busson, D. (1998). Modulation of Hedgehog target gene expression by the Fused serine-threonine kinase in wing imaginal discs. Mech. Dev. *78*, 17–31.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., and Kornberg, T.B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell *89*, 1043–1053.

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature *368*, 208–214.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., and Gelbart, W.M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disc expression of decapentaplegic, a member of the TGFβ family in Drosophila. Development *111*, 657–666.

Blair, S.S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. Development *115*, 21–33.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. Cell *87*, 553–563.

Chen, Y., Gallaher, N., Goodman, R.H., and Smolik, S.M. (1998). Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus. Proc. Natl. Acad. Sci. USA *95*, 2349–2354.

Dominguez, M., Brunner, M., Hafen, E., and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein Cubitus interruptus. Science *272*, 1621–1625.

Eaton, S., and Kornberg, T.B. (1990). Repression of ci-D in posterior compartments of Drosophila by engrailed. Genes Dev. *4*, 1068–1077.

Hammerschmidt, M., Brook, A., and McMahon, A.P. (1997). The world according to hedgehog. Trends Genet. *13*, 14–21.

Hepker, J., Wang, Q.T., Motzny, C.K., Holmgren, R., and Orenic, T.V. (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. Development *124*, 549–558.

Ingham, P.W. (1998). Transducing Hedgehog: the story so far. EMBO J. *17*, 3505–3511.

Lawrence, P.A., and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from Drosophila? Cell *85*, 951–961.

Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., and Cohen, S.M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. Nature *381*, 387–393.

Lee, J.J., von Kessler, D.P., Parks, S., and Beachy, P.A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog: Cell *71*, 33–50.

Locke, J., Rairdan, G., McDermid, H., Nash, D., Pilgrim, D., Bell, J., Roy, K., and Hodgetts, R. (1996). Cross-screening: a new method to assemble clones rapidly and unambiguously into contigs. Genome Res. *6*, 155–165.

Marigo, V., Johnson, R.L., Vortkamp, A., and Tabin, C.J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev. Biol. *180*, 273–283.

Monnier, V., Dussillol, F., Alves, G., Lamour-Isnard, C., and Plessis, A. (1998). Molecular interactions between three members of the Drosophila Hedgehog signaling pathway: Suppressor of fused links the ser-thr kinase Fused and the transcription factor Cubitus Interruptus. Curr. Biol. *8*, 583–586.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a Dpp morphogen gradient. Cell *85*, 357–368.

Neumann, C., and Cohen, S. (1997). Morphogens and pattern formation. Bioessays *19*, 721–729.

Ohlmeyer, J.T., and Kalderon, D. (1998). Hedgehog stimulates the maturation of Cubitus interruptus into a labile transcriptional activator. Nature *396*, 749–753.

Orenic, T.V., Slusarski, D.C., Kroll, K.L., and Holmgren, R.A. (1990). Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila. Genes Dev. *4*, 1053–1067.

Pignoni F., and Zipursky, S.L. (1997). Induction of Drosophila eye development by decapentaplegic. Development *124*, 271–278.

Préat, T., Thérond, P., Limbourg-Bouchon, B., Pham, A., Tricoire, H., Busson, D., and Lamour-Isnard, C. (1993). Segmental polarity in Drosophila melanogaster: genetic dissection of fused in a Suppressor of fused background reveals interaction with costal-2. Genetics *135*, 1047–1062.

Robbins, D.J., Nybakken, K.E., Kobayashi, R., Sisson, J.C., Bishop, J.M., and Therond, P.P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. Cell *90*, 225–234.

Sanchez-Herrero, E., Couso, J.P., Capdevila, J., and Guerrero, I. (1996). The fu gene discriminates between pathways to control dpp expression in Drosophila imaginal discs. Mech. Dev. *55*, 159–170.

Sanicola, M., Sekelsky, J., Elson, S., and Gelbart, W.M. (1995). Drawing a stripe in Drosophila imaginal disks: negative regulation of decapentaplegic and patched expression by engrailed. Genetics *139*, 745–756.

Schweizer, L., and Basler, K. (1998). Drosophila Ci^D encodes a hybrid Pangolin/Cubitus Interruptus protein that diverts the Wingless into the Hedgehog signaling pathway. Mech. Dev. *78*, 141–151.

Sisson, J.C., Ho, K.S., Suyama, K., and Scott, M.P. (1997). Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. Cell *90*, 235–245.

Slusarski, D.C., Motzny, C.K., and Holmgren, R. (1995). Mutations that alter the timing and pattern of cubitus interruptus gene expression in Drosophila melanogaster. Genetics *139*, 229–240.

van den Heuvel, M., and Ingham, P.W. (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signaling. Nature *382*, 547–551.

Von Ohlen, T., and Hooper, J.E. (1997). Hedgehog signaling regulates transcription through Gli/Ci binding sites in the wingless enhancer. Mech. Dev. *68*, 149–156.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. Development *117*, 1223–1237.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. Development *121*, 2265–2278.